THE AMINO ACID SEQUENCE OF AMPHOMYCIN

I. THE "CORE-HEXAPEPTIDE"

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A hexapeptide with the amino acid sequence glycyl-D-*erythro*-α, β-diaminobutyryl-L-valyl-L-prolyl-(L-*threo*-α, β-diaminobutyric acid, D-pipecolic acid) was obtained by selective acid hydrolysis of amphomycin.

Selective hydrolysis of amphomycin with boiling 0.03 N hydrochloric acid[1] resulted in the liberation of the fatty acid residues[2] which acylate the N-terminal moiety*. Three moles of L-aspartic acid[3], one mole of L-*threo*-β-methylaspartic acid[4] and one mole of glycine were also found to be present in the mixture. From the method of cleavage[1], it is obvious that the glycine originates from a residue between two aspartyl moieties (or one aspartyl and the β-methylaspartyl residue) in the sequence of the antibiotic. The remaining constituents[3], one mole each of glycine, L-proline, L-valine, D-pipecolic acid, L-*threo*-α, β-diaminobutyric acid, and D-*erythro*-α, β-diaminobutyric acid[5], were found in a hexapeptide which could be purified by ion-exchange chromatography or by preparative paper chromatography and was isolated in larger amounts by countercurrent distribution. From the conditions of the hydrolysis, it can be deduced that in the sequence of the parent molecule, the hexapeptide is both preceded and followed by aspartic acid (or β-methylaspartic acid). Therefore, this fragment is the central part of the amphomycin molecule and will be called the "core-hexapeptide" in this paper**.

From its amino acid composition, it seemed highly probable that the core-hexapeptide of amphomycin is identical with one of the fragments obtained by FUJINO[6] in the partial acid hydrolysis of glumamycin. To exclude the remote possibility that the two antibiotics, amphomycin and glumamycin, have identical amino acid compositions but with the residues arranged in different sequences, a more detailed study of the hexapeptide was necessary. Furthermore, in the structural studies on

* Hydrolysis with boiling 0.25 N acetic acid for 1 hour also cleaves amphomycin. An ether-extractable material, L-aspartic acid acylated by fatty acids, was isolated from the hydrolysate. This is the N-terminal part of the antibiotic.

** Under identical conditions, the same fragment is liberated from aspartocin (cf. ref. 8).

glumamycin[7], the two α,β-diaminobutyric acid constituents were considered to have the same stereochemistry. This conclusion seems to be erroneous: if for no other reasons, because of racemization during hydrolysis[5] the presence of two diastereoisomers must be postulated in the hydrolysis mixture, even if only one of these would participate as a constituent in the parent molecule. Our studies[5] demonstrated that, in amphomycin, one of the α,β-diaminobutyric acid residues has the D-*erythro* (^{e}Dab), the other the L-*threo* configuration (^{t}Dab). The lack of differentiation between the isomers rendered the otherwise elegant work[6] on glumamycin incomplete and necessitated a new effort toward the sequence of the core-hexapeptide. The results of these investigations add support to our contention[8] that glumamycin and amphomycin are identical.

The N-terminal residue of the core-hexapeptide was shown, both by dansylation[9] and by EDMAN degradation[10], to be glycine. It was less easy to assign a sequence to the other five amino acids. No method was found for a selective cleavage of the hexapeptide, and the presence of the two α,β-diaminobutyric acid residues further complicated the determination of sequence: *e.g.*, the EDMAN method, after one cycle, failed to give useful information. An attempt was made to elucidate the sequence through identification of the diketopiperazines (DKPs) formed in the pyrolysis of the hexapeptide. The presence of the DKP |—Val-Pro—| could be firmly established by the characteristic mass spectrum, especially with chemical ionization. The second major component, the DKP of pipecolic acid (Pip) and dehydrobutyrine (Dhb) obviously originates from the DKP |—Pip-Dab—|. The next-neighbor relationship (Pro, Val) and (Dab, Pip) were insufficient for the assignment of a sequence even when considered together with the earlier found[11] next-neighbor relationship (^{e}Dab, Val)* and with the knowledge that glycine is the N-terminal. Therefore, a reexamination of the

Table 1. High-resolution mass spectrometric data of the "core-hexapeptide" [a]

Mass found	Int. (%)	Mass calcd.	Elemental comp.	Interpretation
438.238	0.7	438.235	$C_{20}H_{32}N_5O_6$	Ac(Gly, Ac-Dab, Val, Pro)
410.248	0.4	410.240	$C_{19}H_{32}N_5O_5$	〃 -CO
379.197	0.3	379.198	$C_{18}H_{27}N_4O_5$	〃 -acetamide
322.182	0.6	322.177	$C_{16}H_{24}N_3O_4$	〃 -acetylglycinamide
341.185	7.0	341.183	$C_{15}H_{25}N_4O_5$	Ac(Gly, Ac-Dab, Val)
313.187	2.7	313.188	$C_{14}H_{25}N_4O_4$	〃 -CO
282.149	4.1	282.145	$C_{13}H_{20}N_3O_4$	〃 -acetamide
225.123	2.7	225.124	$C_{11}H_{17}N_2O_3$	〃 -acetylglycinamide
242.114	11.0	242.114	$C_{10}H_{16}N_3O_4$	Ac(Gly, Ac-Dab)
214.118	4.4	214.119	$C_9H_{16}N_3O_3$	〃 -CO
183.077	2.3	183.077	$C_8H_{11}N_2O_3$	〃 -acetamide
126.054	0.5	126.055	$C_6H_8NO_2$	〃 -acetylglycinamide
100.040	3.1	100.040	$C_4H_6NO_2$	Ac-Gly
72.045	20.6	72.045	C_3H_6NO	〃 -CO
194.105	100	194.105	$C_{10}H_{14}N_2O_2$	|—Pip-Dhb—|

a) After acetylation and treatment with diazomethane.

* The tentative squence[11] of valine and D-*erythro*-α,β-diaminobutyric acid had to be revised. The formation of several products through rearrangements during the slow hydrolysis[11] made this assignment difficult.

sequence with the aid of high-resolution mass spectra (HRMS) was decided upon.

The core-peptide was acetylated with acetic anhydride in acetic acid and the product treated, in methanol, with an ethereal solution of diazomethane. Data of the HRMS are listed in Table 1. The observed masses gave sufficient evidence on the order (Gly-Dab-Val-Pro-) of the first four amino acids in the sequence of the core-hexapeptide, but not enough information was obtained about the C-terminal part, most of which appeared in the form of |—Dhb-Pip—| (mass 194). Because of the remaining ambiguity, the HRMS of an acetylated-permethylated[12,13] sample were also examined. The pertinent data of this spectrum are summarized in Table 2. Finally, a sample of the acetyl-hexapeptide was permethylated with CD_3I[14,15]. The HRMS (Table 3) was particularly clear. Nevertheless, the spectra obtained on permethylated samples merely confirmed the sequence of the first four residues in the core-hexapeptide. The conclusions from the HRMS study are summarized in sequence I.

Table 2. High-resolution mass spectrometric data. Acetylated-permethylated "core-hexapeptide"

Mass found	Int. (%)	Mass calcd.	Elemental Comp.	Interpretation [a]
508.317	3.3	508.314	$C_{25}H_{42}N_5O_6$	Ac. (Ala[b]), Ac-Dab, Val, Pro)
494.298	17.9	494.298	$C_{24}H_{40}N_5O_6$	Ac. (Gly, Ac-Dab, Val, Pro)
466.299	1.8	466.302	$C_{23}H_{40}N_5O_5$	〃 -CO
435.261	0.5	435.261	$C_{22}H_{35}N_4O_5$	Ac. (Ala[b]), Dhb, Val, Pro)
421.253	0.8	421.245	$C_{21}H_{33}N_4O_5$	Ac. (Gly, Dhb, Val, Pro)
350.209	8.6	350.208	$C_{18}H_{28}N_3O_4$	Ac. (Dhb, Val, Pro)[c]
411.262	1.5	411.261	$C_{20}H_{35}N_4O_5$	Ac. (Ala[b]), Ac-Dab, Val)
397.246	14.6	397.245	$C_{19}H_{33}N_4O_5$	Ac. (Gly, Ac-Dab, Val)
369.245	1.2	369.250	$C_{18}H_{33}N_4O_4$	〃 -CO
324.193	10.3	324.192	$C_{16}H_{26}N_3O_4$	Ac. (Gly, Dhb, Val)
310.179	1.1	310.177	$C_{15}H_{24}N_3O_4$	Ac. (Gly, Dhb, Val[d])
253.155	59.3	253.155	$C_{13}H_{21}N_2O_3$	Ac. (Dhb, Val)
298.177	3.0	298.177	$C_{14}H_{24}N_3O_4$	Ac. (Ala[b]), Ac-Dab)[e]
284.165	12.3	284.161	$C_{13}H_{22}N_3O_4$	Ac. (Gly, Ac-Dab)
256.165	1.4	256.166	$C_{12}H_{22}N_3O_3$	〃 -CO
225.125	3.5	225.124	$C_{11}H_{17}N_2O_3$	Ac. (Ala[b]), Dhb)[f]
211.107	6.8	211.108	$C_{10}H_{15}N_2O_3$	Ac. (Gly, Dhb)
183.113	7.5	183.113	$C_9H_{15}N_2O_2$	〃 -CO
140.073	44.9	140.071	$C_7H_{10}NO_2$	Ac-Dhb
128.071	16.2	128.071	$C_6H_{10}NO_2$	Ac-Ala[b,g]
114.055	100.0	114.055	$C_5H_8NO_2$	Ac-Gly
100.076	17.2	100.076	$C_5H_{10}NO$	Ac-Ala-CO
86.060	12.4	86.061	C_4H_8NO	Ac-Gly-CO
208.119	2.14	208.121	$C_{11}H_{16}N_2O_2$	|—Dhb-Pip—|

a) With the exception of Pro and Pip, all the residues are considered N-methylated; b) From C-methylation of Gly; c) 494-acetylsarcosine methylamide; d) Val, not permethylated; e) Could be (Pro, Ac-Dab)·OCH_3; f) Corresponds also to (Pro, Dhb)·OCH_3; g) Could be interpeted as Dhb·OCH_3.

Table 3. High-resolution mass spectrometric data. Acetylated-perdeuteriomethylated "core-hexapeptide"

Mass found	Int. (%)	Mass calcd.	Elemental comp.	Interpretation*
506.375	2.7	506.373	$C_{24}H_{28}D_{12}N_5O_6$	Ac(Gly, Dab, Val, Pro)
409.321	5.2	409.320	$C_{19}H_{21}D_{12}N_4O_5$	Ac(Gly, Dab, Val)
333.252	18.4	333.249	$C_{16}H_{17}D_9N_3O_4$	Ac(Gly, Dhb, Val)
293.221	7.2	293.217	$C_{13}H_{13}D_9N_3O_4$	Ac(Gly, Dab)
217.146	18.1	217.146	$C_{10}H_9D_6N_2O_3$	Ac(Gly, Dhb)
189.155	8.3	189.151	$C_9H_9D_6N_2O_2$	〃 -CO
143.089	8.7	143.090	$C_7H_7D_3NO_2$	Ac-Dhb
131.085	5.2	131.090	$C_6H_7D_3NO_2$	Ac-Ala**
117.074	61.1	117.074	$C_5H_5D_3NO_2$	Ac-Gly
103.095	31.0	103.095	$C_5H_7D_3NO$	Ac-Ala-CO
89.080	18.5	89.079	$C_4H_5D_3NO$	Ac-Gly-CO
211.141	7.9	211.141	$C_{11}H_{13}D_3N_2O_2$	|—Pip-Dhb—|

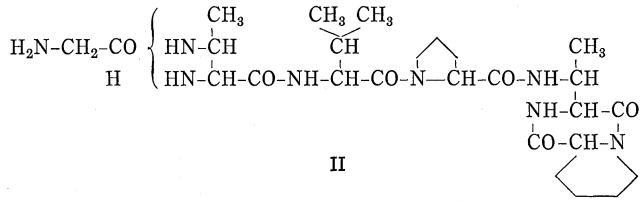
* With the exception of Pro and Pip, all residues were considered as N-deuteriomethylated. Dab is understood as acetylated.
** From C-methylation of Gly.

Gly-Dab-Val-Pro-(Dab, Pip)

I

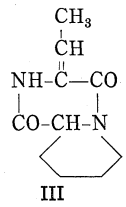
Considerable new light was shed on the structure of the core-hexapeptide by elemental analysis and titration data. The analytical values fitted the formula $C_{26}H_{46}N_8O_7 \cdot 2CH_3COOH \cdot 1.5H_2O$ or rather, as it will be shown below, $C_{26}H_{44}N_8O_6 \cdot 2CH_3COOH \cdot 2.5H_2O$. The two moles of acetic acid could be titrated with alkali (neut. equiv. 369). Titration with perchloric acid gave a neut. equiv. of 364, thus revealing two rather than three basic centers. Also, the high mobility of the core-peptide on paper chromatograms and thin-layer chromatograms was quite remarkable.

The conflict between these observations and the expected properties of a peptide with sequence I could be reconciled by assuming structure II for the core-peptide:



II

The presence of the DKP partial structure in II would diminish the number of free, ionizable groups in I from 4 to 2 and would correspondingly result in higher mobility on paper chromatograms. Furthermore, in structure II, indeed only two basic centers are available for titration with perchloric acid, while in a straight chain structure with sequence I, three basic groups should be present. Finally, structure II can fully explain the absence of the C-terminal methyl esters from the HRMS and also the presence, both among the products of pyrolysis and in the mass spectra, of the DKP |—Pip-Dhb—| (III), the formation of which from II by elimination is not unexpected*.



III

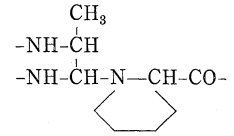
To test the absence of a free carboxyl group, the compound was subjected to electrophoresis on paper at pH 10. The core-peptide migrated toward the cathode. This crucial experiment strongly supports structure II.

From the diketopiperazine arrangement in II, it follows directly that the prolyl residue is attached to the β- rather than α-amino group of the D-*threo*-α,β-diaminobutyryl residue. Treatment of the core-hexapeptide with nitrous acid led to the destruction of the glycine and D-*erythro*-α,β-diaminobutyric acid residues with the concomitant appearance of threonine. This finding would suggest that the glycine is

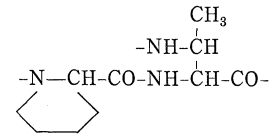
* Partial acid hydrolysis (1 N HCl, 100°C, 4 hours) of the hexapeptide yielded a ninhydrin-positive fragment, Rf 0.54, which on complete hydrolysis gave equimolar amounts of Pip and ^{t}Dab. The mobility in the butanol-acetic acid-pyridine-water system indicates the DKP |—Pip-^{t}Dab—|. Furthermore, the HRMS of the hydrolysate, after acetylation and methylation (with diazomethane), revealed mass 194.109, corresponding to the DKP |—Pip-Dhb—| (calcd. for $C_{10}H_{14}N_2O_2$: 194.106), as one of the intense peaks.

linked to the α-amino group of ^{e}Dab, but the rather low yield of threonine (cf. experimental part) renders the evidence from the deamination experiment questionable. It is expected that this remaining ambiguity will be removed when the sequence study is extended beyond the hexapeptide fragment.

It should be emphasized that the diketopiperazine in **II** is not necessarily a genuine feature of the antibiotic. It is quite conceivable that the ring closure occurs during the long hydrolysis with weak acid, conditions applied for the removal of the aspartyl and methylaspartyl residues from the molecule of amphomycin. If this is the case, then the DKP part of the core-hexapeptide could originate either from partial structure **IV** or from **V**. We hope that our present studies will clarify this question.



IV



V

Experimental

For paper chromatography, Whatman No. 3MM papar and the solvent system *n*-butanol – acetic acid – pyridine – water (30 : 6 : 20 : 24) were used (descending).

For quantitative amino acid analysis, samples were hydrolyzed with constant boiling hydrochloric acid in evacuated, sealed ampoules, at 110°C for 16 hours. After removal of the acid by evaporation, the analyses were carried out with the aid of a Beckman-Spinco 120C amino acid analyzer.

Partial acid hydrolysis of amphomycin and separation of the products: (**a**) Purified[3] amphomycin (amphoter, 500 mg) was dissolved in 0.03 N HCl (100 ml) and heated under reflux for 34 hours. After extraction with ether (2×100 ml), the solution was evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in a small volume of water and applied to a column (1×10 cm) of Dowex 1×8 in acetate cycle. The neutral and basic products were eluted with water. The acidic materials were recovered from the column with 2 N acetic acid and were identified as aspartic acid and β-methylaspartic acid. After evaporation to dryness of the fractions containing the neutral and basic products, the residue was dissolved in a small amount of water and applied to a column (1×10 cm) of Dowex 50W-X12 in NH_4^+ cycle. The neutral material was eluted with water and was shown to be glycine. The column was then eluted with 1 % NH_4OH; fractions of 3 ml were collected and were examined by thin-layer chromatography in the system *n*-butanol – acetic acid – water (3 : 1 : 1) and by paper electrophoresis at pH 3.5 and 6.5 (30 volts/cm). Fractions no. 12~32 contained a homogeneous product by these criteria. This compound (82 mg) was found to have the following amino acid composition: Pro, 1.02; Gly, 0.97; Val, 0.92; Pip, 1.07; Dab, about 2.

(**b**) Amphomycin amphoter (6 mg) was hydrolyzed with 0.03 N hydrochloric acid (3 ml) in two evacuated, sealed ampoules at 110°C for 16 hours. After evaporation of the acid with a stream of nitrogen, the residue was applied on a full sheet of Whatman 3MM paper and chromatographed (descending) in the solvent system *n*-butanol – acetic acid – pyridine – water (30 : 6 : 24 : 20). After locating the core-peptide on guide strips with ninhydrin, the corresponding band (Rf 0.43) was eluted with 30 % acetic acid. The solvent was evaporated with a stream of nitrogen. Quantitative amino acid analysis gave the following ratios: Pro, 1.0; Gly, 1.0; Val, 0.75; Pip, 0.9; Dab, 1.7 (The Dab-Val bond is not completely hydrolyzed in the usual 16-hour hydrolysis[11]).

(**c**) The neutral and basic products (obtained from 1.5 g amphomycin, as described

under a) were dissolved in *ca.* 10 ml of the lower layer of the solvent system, *n*-butanol – acetic acid – pyridine – water (4 : 1 : 3 : 7) and the solution was placed into the first four tubes of a 520-tnbe automatic CRAIG apparatus (3-ml phases). After 520 transfers, the single withdrawal technique was followed. After 1,000 transfers, no more upper layer was added, but the distribution was continued until all tubes were free from upper phase. The core-hexapeptide was located with the help of paper chromatograms and was collected from tubes No. 231~270. The contents of these tubes were pooled, the solvents were removed by evaporation with a stream of nitrogen, and the residue was dried *in vacuo* at room temperature (0.3 g).

For analysis, a sample was dried at 45°C and 0.05 mm for 2 hours. Paper chromatograms indicated no change in the material due to the conditions of drying.

Anal. Calcd for $C_{26}H_{44}N_8O_6 \cdot 2CH_3COOH \cdot 2.5H_2O$ (m.w. 724.85): C 49.37, H 7.87, N 15.35. Neut. equiv. (assuming two basic and two acidic centers): 365. Found: C 49.26, H 7.46, N 15.47. Neut. equiv. 364 (titrated with $HClO_4$): 369 (with NaOH).

In the IR spectrum, no ester or lactone CO bands were observed. The UV spectrum shows only end absorption.

On paper chromatograms, the hexapeptide traveled with an Rf value of 0.43, while a synthetic hexapeptide with the amino acid composition (Scr, Pro, Gly, Ala, Lys, Arg) had an Rf of 0.05. On precoated silica gel thin-layer plates (Merck), in the system *n*-butanol – acetic acid – water (4 : 1 : 1), the synthetic peptide remained at the origin, while the core-hexapeptide moved with an Rf value of 0.15. Both on paper and on silica gel, the core-peptide gave a greenish-yellow color with ninhydrin. The color gradually turned into dark greyish purple.

Electrophoresis was carried out on the flat plate of a Savant high-voltage electrophoresis apparatus. In pyridine – acetic acid – water (25 : 1 : 225) at pH 6.5, 2,000 v, 1 hour, the peptide traveled 5.2 cm toward the cathode (The above-mentioned synthetic peptide traveled the same distance, lysin 6.8 cm, leucine remained at the origin, aspartic acid moved 8.4 cm towards the anode). In 2 N acetic acid at 2,200 v and 1.5 hours, the core-hexapeptide traveled 16.3 cm towards the cathode: the control-synthetic hexapeptide moved 16.2 cm (arginine 19 cm). In 0.05 M borate buffer, adjusted with 2 N NaOH to pH 10 at 3,000 v and 45 minutes, the core-peptide moved 2 cm toward the cathode, while the Dab sample used for comparison moved 1.8 cm toward the anode.

Determination of the N-terminal residue of the hexapeptide by dansylation (6): A sample (1 mg) of the hexapeptide was dissolved in 0.25 % $NaHCO_3$ (0.3 ml), and a 2.5 % solution (0.3 ml) of dansylchloride in acetone was added. After 30 minutes at room temperature, the solvents were removed with a stream of nitrogen, the residue was dissolved in 6 N HCl and hydrolyzed in an evacuated and sealed ampoule at 110°C for 4 hours. After removal of the hydrochloric acid, the residue was chromatographed on thin-layer plates of silica gel in a system of chloroform – benzyl alcohol – acetic acid (30 : 10 : 1). Through comparisons with authentic samples of dansylamino acids, the N-terminal residue of the hexapeptide could be identified as glycine.

Determination of the N-terminal residue of the hexapeptide by EDMAN degradation (7): To a sample (12 mg) of the hexapeptide dissolved in 50 % aqueous pyridine (0.2 ml), a 20 % solution (0.1 ml) of phenylisothiocyanate in pyridine was added. After 4 hours at 50°C (under nitrogen), the solution was evaporated to dryness and the residue dissolved in anhydrous trifluoroacetic acid. After 4 hours at 50°C (under nitrogen), the trifluoroacetic acid was removed *in vacuo* and the residue was treated with 0.03 N HCl (2 ml) at 50°C for 15 minutes. The mixture was extracted with *n*-butylacetate (3×1 ml) and the aqueous layer evaporated to dryness. A sample of this material was hydrolyzed for quantitative amino acid analysis, as described for the hexapeptide, and was found to contain the constituents of the hexapeptide but in the following ratios: Pro, 1.00; Gly, 0.28; Val, 0.85; Pip, about 1; ^{t}Dab, 1.15; ^{e}Dab, 0.78.

The butyl acetate extracts were evaporated to dryness and the residue compared on thin-layer plates of silica gel, with chloroform as solvent, with authentic samples of phenylthiohydantoins derived from amino acids. Spots were revealed with an iodine azide starch reagent[16]. In this way, glycine was identified as the N-terminal residue of the hexapeptide. The identity of the phenylthiodantoin was confirmed by exposure to NH_3 vapors[16] : a red color developed.

Pyrolysis : Samples of about 10 mg were placed in a small test tube (6×25 mm) and placed into a sublimation tube (10×250 mm). The latter was evacuated to about 0.03 mm and heated at one end in an electric oven. The pyrolysate that formed between 200° and 260°C was collected from the unheated part of the sublimation tube with chloroform. In HRMS of the sublimed material, the DKPs |—Pro-Val—| (found, 196.120 ; calcd. for $C_{10}H_{16}N_2O_2$, 196.121), |—Pip-Dhb—| (found, 194.104 ; calcd. for $C_{10}H_{14}N_2O_2$, 194.105) and |—Pro-Gly—| (found, 154.074 ; calcd. for $C_7H_{10}N_2O_2$, 154.074) could be recognized. The last mentioned DKP is a product of the side-chain fragmentation of |—Pro-Val—|. This was shown by the HRMS of authentic |—Pro-Val—| and also by the abundant mass 197 in spectra obtained by chemical ionization. In this method, the DKP |—Pip-Dhb—| appeared as mass 195.

For HRMS, permethylation of the acetyl derivative was carried out according to AGARWAL, KENNER and SHEPPARD[12] and repeated by the procedure of BENOITON and COGGINS[13]. In the treatment with CD_3I, the method of DAS, GÉRO, LEDERER[14] and THOMAS, ITO[15] was followed. High-resolution mass spectral data on the derivatized hexapeptide samples were obtained using an A.E.I. MS-9 mass spectrometer coupled to a digital data system. The samples were introduced into the mass spectrometer via the direct insertion probe at temperatures between 200° and 250°C.

Deamination of the hexapeptide : A sample (7 mg) of the hexapeptide in 0.1 N HCl (1 ml) was treated with N_2O_3 until no more ninhydrin reaction could be observed. The NaN_2O_3 was generated from a $NaNO_2$ and 6 N HCl and was introduced into the solution with the aid of a slow stream of nitrogen. The reaction was carried out at room temperature and required about 3 hours. After evaporation to dryness, the residue was dissolved in N HCl (8 ml) and evaporated on a steam bath under a stream of nitrogen. An aliquot was hydrolyzed for amino acid analyses in the usual way. Thr, 0.29 ; Pro, 1.0 ; Gly, 0.04 ; Val, 1.0 ; Pip, 1.0 ; Dab (mainly *threo*), 1.1.

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